

Enhancing blast disease resistance by overexpression of the calcium-dependent protein kinase *OsCPK4* in rice

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Summary

Rice is the most important staple food for more than half of the human population, and blast disease is the most serious disease affecting global rice production. In this work, the isoform *OsCPK4* of the rice calcium-dependent protein kinase family is reported as a regulator of rice immunity to blast fungal infection. It shows that overexpression of *OsCPK4* gene in rice plants enhances resistance to blast disease by preventing fungal penetration. The constitutive accumulation of *OsCPK4* protein prepares rice plants for a rapid and potentiated defence response, including the production of reactive oxygen species, callose deposition and defence gene expression. *OsCPK4* overexpression leads also to constitutive increased content of the glycosylated salicylic acid hormone in leaves without compromising rice yield. Given that *OsCPK4* overexpression was known to confer also salt and drought tolerance in rice, the results reported in this article demonstrate that *OsCPK4* acts as a convergence component that positively modulates both biotic and abiotic signalling pathways. Altogether, our findings indicate that *OsCPK4* is a potential molecular target to improve not only abiotic stress tolerance, but also blast disease resistance of rice crops.

Keywords: rice, blast, calcium-dependent protein kinases, defence, resistance, productivity.

Introduction

Rice blast disease, caused by the filamentous ascomycete fungus *Magnaporthe oryzae*, is the most important rice disease due to its severity and wide distribution (approximately 85 countries around the world) (Ou, 1987). *Magnaporthe oryzae* attacks rice plants at all developmental stages, more often during the seedling stage, and it can infect leaves, stems, nodes, collars and panicles (Dean *et al.*, 2012). Rice blast causes severe crop losses varying from 10 to 85% depending on the area and climatology (Skamnioti and Gurr, 2009) (<http://www.irri.org/research/better-rice-varieties/disease-and-pest-resistance-in-rice>). Resistant cultivars and pesticides have traditionally been used to control this disease. However, the fungus *M. oryzae* overcomes host resistance quickly, and resistant cultivars become ineffective after a few years (Lee *et al.*, 2009). Pesticide use, on the other hand, is costly and environmentally unfriendly. Being rice a paramount source of human food, new strategies providing long-term blast protection should therefore be developed. The study of the plant defence responses offers a vast field of possibilities to improve disease resistance in rice.

In addition to structural barriers and preformed antimicrobial compounds, plants have evolved inducible immune responses to defend themselves against pathogen attack. The defence response starts with the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) that activate the PAMP-triggered immunity (PTI) (Boller and He, 2009; Chisholm *et al.*, 2006; Jones and Dangl, 2006). Successful pathogens have evolved to suppress the PTI response by the action of effectors. But plants in turn have evolved a second defence layer, known as effector-triggered immunity (ETI),

consisting of resistance proteins that recognize these effectors (Jones and Dangl, 2006). Both PTI and ETI counteract the pathogen attack by inducing immune responses (Tsuda and Katagiri, 2010). The earliest defence reactions include changes in ion fluxes across membranes, an increase in the intracellular calcium concentration, the activation of protein kinases or the synthesis of reactive oxygen species (ROS) (Baxter *et al.*, 2013; Lecourieux *et al.*, 2006; Meng and Zhang, 2013; Seybold *et al.*, 2014; Tena *et al.*, 2011; Torres, 2010). Forward reactions consist of transcriptional reprogramming, alterations in hormone status and cell wall reinforcement through callose depositions and lignifications and in some cases even by cell death at the site of infection (Liu *et al.*, 2014; Luna *et al.*, 2010; Navarro *et al.*, 2004; Tsuda and Katagiri, 2010). Defence responses locally activated in primary pathogen-infected plant tissues are often extended to distal noninfected tissues, conferring systemic acquired resistance (SAR) (Durrant and Dong, 2004; Ryals *et al.*, 1996). This resistance is long-lasting and effective against secondary attack by unrelated pathogens. SAR is associated with the signal molecule salicylic acid (SA) and the accumulation of pathogenesis-related (PR) proteins that are thought to contribute to resistance (Durrant and Dong, 2004).

Calcium influx is one of the earliest events upon pathogen recognition in plant defence response (Ranf *et al.*, 2011). Alterations in calcium concentration are sensed by calcium-binding proteins, including calmodulin, calcium-dependent protein kinases (CDPK or CPKs) and calcineurin B-like proteins, which relay the calcium signal into specific cellular and physiological responses (Dodd *et al.*, 2010; Harper *et al.*, 2004). CPKs represent unique calcium sensors able to translate calcium signals directly into phosphorylation events, because they combine in a

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single molecule a calcium-binding domain and a serine/threonine kinase domain (Harper *et al.*, 2004). In this sense, genetic and biochemical studies have demonstrated that these plant proteins are important players in numerous signalling pathways and biological processes, including stress signalling cascades and immune signalling responses (Boudsocq and Sheen, 2013; Romeis and Herde, 2014; Schulz *et al.*, 2013).

CPKs are encoded by large gene families, the rice genome containing 31 *CPK* genes (Asano *et al.*, 2005; Ray *et al.*, 2007). In contrast to *Arabidopsis* CPKs, little is known about the functions of specific rice CPKs. Among the ones functionally characterized are the *OsCPK13* (Saijo *et al.*, 2000), *OsCPK12* (Asano *et al.*, 2012) and *OsCPK9* (Wei *et al.*, 2014) proteins that have been reported as signalling components of abiotic stress responses; the *OsCPK10* (Fu *et al.*, 2013) and the *OsCPK18* (Xie *et al.*, 2014) were described as positive and negative regulators of *M. oryzae* resistance, respectively. Only *OsCPK12* has been shown to be involved in both abiotic and biotic stress signalling (Asano *et al.*, 2012). Recently, our group reported that *OsCPK4* positively regulates salt and drought stress adaptation (Campo *et al.*, 2014). Contrary to *OsCPK12* that oppositely modulates the different signalling pathways, the present study reports that *OsCPK4* is also a positive regulator of immunity in rice. *OsCPK4* overexpression confers an enhanced resistance to blast disease in rice plants by preventing *M. oryzae* fungal penetration. The enhanced resistance phenotype is associated with the constitutive accumulation of conjugated SA and callose, and a fast and stronger activation of defence responses, including ROS production and defence gene expression, without compromising rice productivity.

Results

OsCPK4 expression is induced by *Magnaporthe oryzae* infection in rice plants

A search for altered expression genes in a microarray-based global transcriptomic analysis of rice plants in response to *M. oryzae* elicitors (Campo *et al.*, 2013) identified the *OsCPK4* gene as an up-regulated gene in leaves after 2-h treatment (fold change = 1.94; *P*-value = 0.0002). The *OsCPK4* gene (LOC_Os02g03410) encodes a CDPK involved in the adaptation of rice plants to salinity and drought conditions (Campo *et al.*, 2014). To confirm that *OsCPK4* gene expression is altered during the defence response of rice plants, it was examined in leaves at different times after inoculation with *M. oryzae* spores (Figure 1a). *OsCPK4* expression was rapid and strongly induced in rice leaves at earlier stages of infection at 6 h postinoculation (hpi), coinciding with the formation of the fungal infective structure, named appressorium (Wilson and Talbot, 2009). *OsCPK4* activation increased until 12 hpi (approximately an eightfold increase) and started to decrease at 24 hpi, once fungal penetration had already occurred. These observations show that *OsCPK4* is an early-response gene against *M. oryzae* infection in rice leaves.

OsCPK4 protein accumulation was also examined in blast-infected leaves. In agreement with *OsCPK4* transcript levels, Western blot analyses showed an increase in the accumulation of the encoded protein after pathogen inoculation (Figure 1b). These results indicate that *OsCPK4* transcriptional activation is translated in the protein accumulation and suggest that the *OsCPK4* protein is involved in the defence response of rice plants to *M. oryzae* infection.

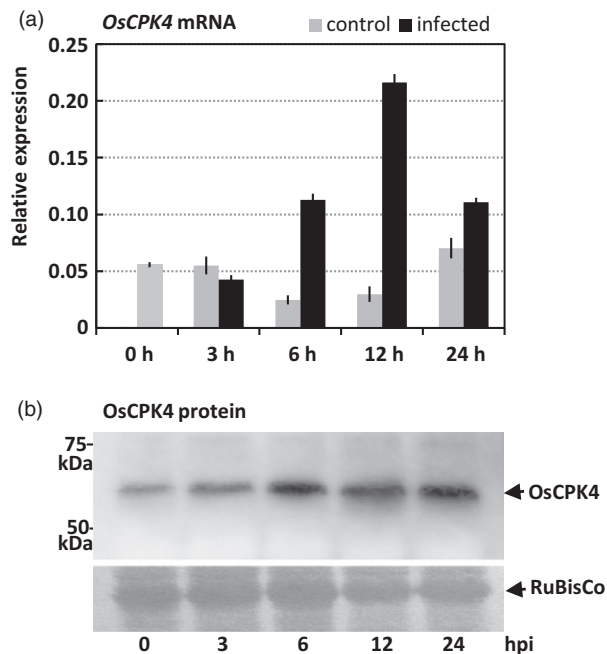


Figure 1 *OsCPK4* expression and protein accumulation in response to fungal infection. (a) Transcript levels were determined by qRT-PCR analysis in rice leaves (*Oryza sativa* cultivar Nipponbare) after inoculation with a *Magnaporthe oryzae* spore suspension (10^5 spores/mL) at the indicated period of time. Specific primers were used to detect the *OsCPK4* mRNA levels that were normalized to the *OsUbi5* mRNAs. Error bars indicate SEM of three replicates. (b) *OsCPK4* accumulation was determined by Western blot analysis using specific anti-*OsCPK4* antibodies at the indicated period of time after inoculation. Lower panel corresponds to Ponceau staining of protein samples (40 μ g per lane). Leaves from three different plants grown in soil for 3 weeks were collected in a pool at each different time for total RNA (a) or protein extraction (b). Results are representative of two independent experiments.

OsCPK4 overexpressor rice plants are more resistant to *Magnaporthe oryzae* infection

To further investigate the function of *OsCPK4* in rice immunity, we used the transgenic *OsCPK4*-overexpressing rice plants previously described (Campo *et al.*, 2014). These plants were produced in the japonica cultivar Nipponbare and expressed the *OsCPK4* full-length cDNA under the control of the strong and constitutive *ZmUbi1* promoter. Quantitative RT-PCR analyses confirmed that the expression of *OsCPK4* was indeed significantly enhanced in the leaves of *OsCPK4*-Ox plants in comparison with wild-type or control empty vector plants (Figure S1a), resulting also in an increased accumulation of the corresponding protein (Figure S1b). The activity of the accumulated protein is dependent on the presence of calcium (Figure S1c), suggesting that it remains as a latent protein in the rice leaves prone to be stimulated by calcium changes.

The phenotype of *OsCPK4*-Ox lines, compared to wild-type or empty vector plants, was then characterized when challenged with the blast fungus using a detached leaf assay (Coca *et al.*, 2004). Following inoculation with the *M. oryzae* virulent strain FR13, the *OsCPK4*-Ox leaves developed less severe disease symptoms than control leaves (Figure 2a). At 7 dpi, extensive necrotic lesions with fungal sporulation were macroscopically observed on wild-type and empty vector leaves, whereas only few

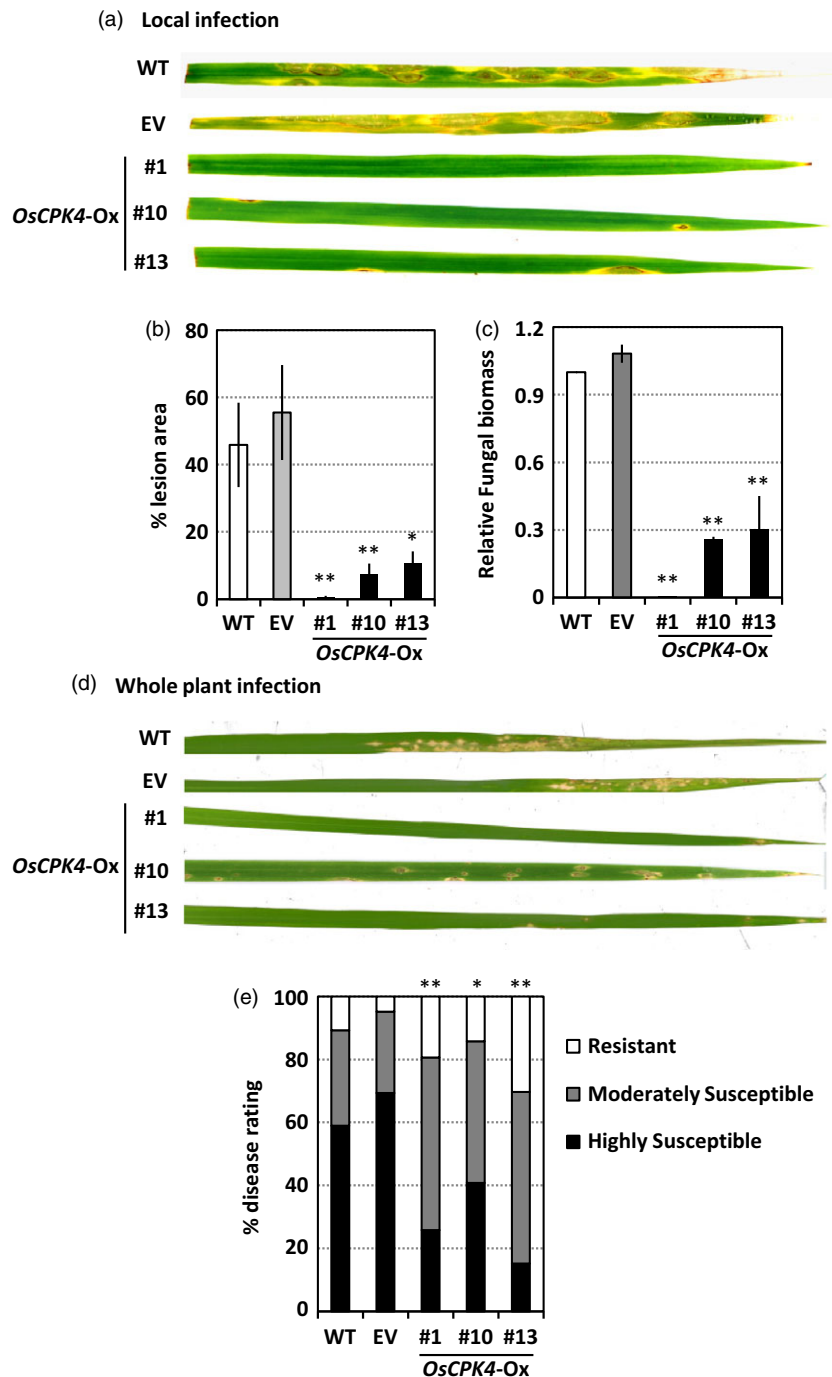


Figure 2 *OsCPK4*-overexpressing plants are more resistant to *Magnaporthe oryzae* infection. (a) Rice disease lesions caused by *M. oryzae* locally inoculated (10^5 spores/mL) on leaves of wild-type (WT), empty vector (EV) and *OsCPK4*-Ox plants (lines #1, #10 and #13) at 7 dpi. (b) Percentage average of lesion area per leaf of three independent assays with three replicates per line at 7 dpi. (c) Relative fungal amount as determined by qPCR of *M. oryzae* 26S rDNA gene compared to *OsUbi1* gene and referred to WT. Values correspond to the average of three independent assays in which three leaves were used for quantification. (d) Disease lesions on leaves from spray-inoculated whole rice plants with *M. oryzae* spore suspension (10^5 spores/mL) at 7 dpi. (e) Disease rating for ten plants per line at 7 dpi following the Standard Evaluation System for blast rice disease (IRRI, 2002) based on leaf lesion area percentage. Mean values of 2 independent assays. Asterisks represent significant differences (one-way ANOVA and Tukey's test; * $P \leq 0.05$, ** $P \leq 0.01$).

lesions were developed on the *OsCPK4*-Ox leaves. The percentage of leaf area affected by blast lesions was determined by image analyses. The results revealed a statistically significant reduction on the lesion area of three independent transgenic lines as compared to control leaves (Figure 2b). In agreement with visual inspection, *OsCPK4*-Ox leaves contained a significant less fungal biomass than control leaves, as determined by qPCR analysis of *M. oryzae* DNA (Figure 2c). The enhanced resistance phenotype to the blast fungus exhibited by *OsCPK4*-Ox leaves was then confirmed by whole-plant infection assays. In this case, rice plants were spray-inoculated with a *M. oryzae* spore suspension, under experimental conditions similar to field conditions. The wild-type

and empty vector control plants developed the typical blast disease lesions, whereas the *OsCPK4*-Ox plants showed clearly less and smaller infection lesions (Figure 2d). Further measure of disease severity showed that a higher percentage of *OsCPK4*-Ox plants exhibited resistant phenotype (around 22%) than wild-type or empty vector plants (around 5–10%), and a lower percentage exhibited highly susceptible phenotype (around 27%) than control plants (65%) (Figure 2e). Collectively, these results suggest that *OsCPK4* positively mediates an enhanced resistance to blast fungal infection.

To gain more insight into the nature of the enhanced blast resistance observed in the *OsCPK4*-Ox plants, the infection

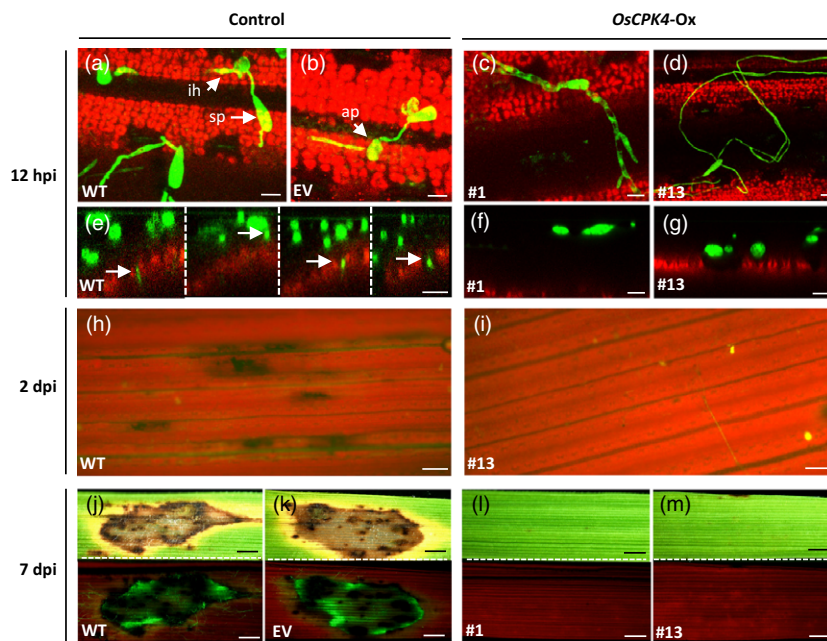


Figure 3 Microscopic analysis of *Magnaporthe oryzae* infection process on rice leaves. Representative images of *OsCPK4* overexpressor (lines 1 and 13), wild-type (WT) and control empty vector (EV) leaves inoculated with the GFP-*M. oryzae* spores (10^5 spores/mL). (a–g) Images of confocal laser microscopy of leaves at 12 hpi, corresponding to projections (a–d) and xz slides (e–g). Epifluorescence images at 2 dpi (h–i) or 7 dpi (j–m, lower panels). Stereoscopic brightfield images. Bars = 10 μ m (a–g), 100 μ m (h–i), 1 mm (j–m). Key: sp, spore; ap, appressorium; ih, invasive hypha.

process and fungal development in rice leaves was investigated by fluorescence microscopy analysis using a GFP-expressing *M. oryzae* virulent strain (GFP-Guy11). GFP expression is reported not to affect the pathogenicity of *M. oryzae* fungal strains (Campos-Soriano and San Segundo, 2009; Sesma and Osbourn, 2004). At early infection stages (12 hpi), *M. oryzae* spores were easily visualized on the leaf surface of the rice plants by fluorescence confocal microscopy (Figure 3a–d). Most of the spores on wild-type and empty vector leaves were germinated and produced short germ tubes that developed appressoria and invasive hyphae penetrating into epidermal cells (Figure 3a–b, e). However, *M. oryzae* spores on *OsCPK4*-Ox leaves germinated freely developing abnormal germ tubes—in some cases thick and highly vacuolated (Figure 3c), while, in others, thin and very long (Figure 3d), without visible evidences of penetration events (Figure 3f–g). These observations support that fungal penetration was impaired in *OsCPK4*-Ox leaves. After 2 dpi, infection lesions were visible under fluorescent microscopy in control leaves (Figure 3h), but not in *OsCPK4*-Ox leaves (Figure 3i). At later stages (7dpi), *M. oryzae* completed its life cycle in wild-type and empty vector leaves showing the typical blast lesions with a bright fluorescent mycelia growing and sporulating (Figure 3j–k). Only small necrotic spots were observed in the *OsCPK4*-Ox leaves (Figure 3l–m). Our observations indicate that *OsCPK4*-mediated resistance relies in the interference with fungal penetration rather than colonization.

The resistance of *OsCPK4*-Ox plants to other rice pathogens was also evaluated. Seedlings were assayed against the seed-borne and soil-transmitted fungal pathogen *Fusarium verticillioides*, which has been associated with the bakanae disease in rice (Wulff *et al.*, 2010). Our results indicate that *OsCPK4*-Ox seedlings are as susceptible to *F. verticillioides* infection as control wild-type and empty vector plants (Figure S2). Similarly, *OsCPK4*-Ox seedlings were equally susceptible as control seedlings when challenged with the bacterial pathogen *Dickeya dadantii*, previously known as *Erwinia chrysanthemi*, the causal agent of foot rot in rice (Goto, 1979; Mansfield *et al.*, 2012). These results suggest that the enhanced resistance to *M. oryzae* shown by *OsCPK4*-Ox

plants is specific against this fungal pathogen and that it does not affect their defence against other rice pathogens with different pathogenesis mechanisms.

Defence response is early activated in *OsCPK4* overexpressor rice plants

One of the earliest defence reactions is the production of ROS, a hallmark of successful pathogen recognition and activation of plant defence response (Torres, 2010). Because *OsCPK4* interferes with the *M. oryzae* infection process at early stages, the ROS production during defence responses in *OsCPK4*-Ox rice leaves was investigated. ROS formation was monitored *in vivo* using the CM-H₂DCFDA probe, a noninvasive fluorescent ROS indicator (Kristiansen *et al.*, 2009). Microscopic analyses showed the induction of fluorescence in rice leaves in response to elicitor treatment, which was faster and stronger in the *OsCPK4* than in wild-type or control empty vector leaves (Figure 4a). Thirty minutes after elicitor treatment, fluorescence was barely visualized in the wild-type or empty vector leaves, but clearly visible in the leaves of two independent *OsCPK4*-Ox lines (Figure 4a, middle panels). At 1-hour treatment, the ROS formation was already detected in the wild-type and empty vector leaves, although a stronger fluorescent labelling was observed in the *OsCPK4* lines (Figure 4a, lower panels). Fluorescence quantification showed significant differences in intensity and timing of ROS formation between *OsCPK4*-Ox and control lines (Figure 4b). Similarly, ROS production was significantly stronger in the *OsCPK4*-Ox leaves compared to control leaves in response to *M. oryzae* spore inoculation (Figure 4c–d). These observations suggest that *OsCPK4* accumulation mediates accelerated and potentiated ROS formation in response to *M. oryzae* infection in rice leaves.

Another defence hallmark is the callose deposition to fortify cell walls that avoids pathogen penetration into the plant cell (Luna *et al.*, 2010; Voigt, 2014). Given that *OsCPK4* overexpression prevents fungal penetration, the callose accumulation was analysed in *OsCPK4*-Ox leaves. Callose was clearly visualized after aniline blue staining as intense blue-green fluorescence under UV

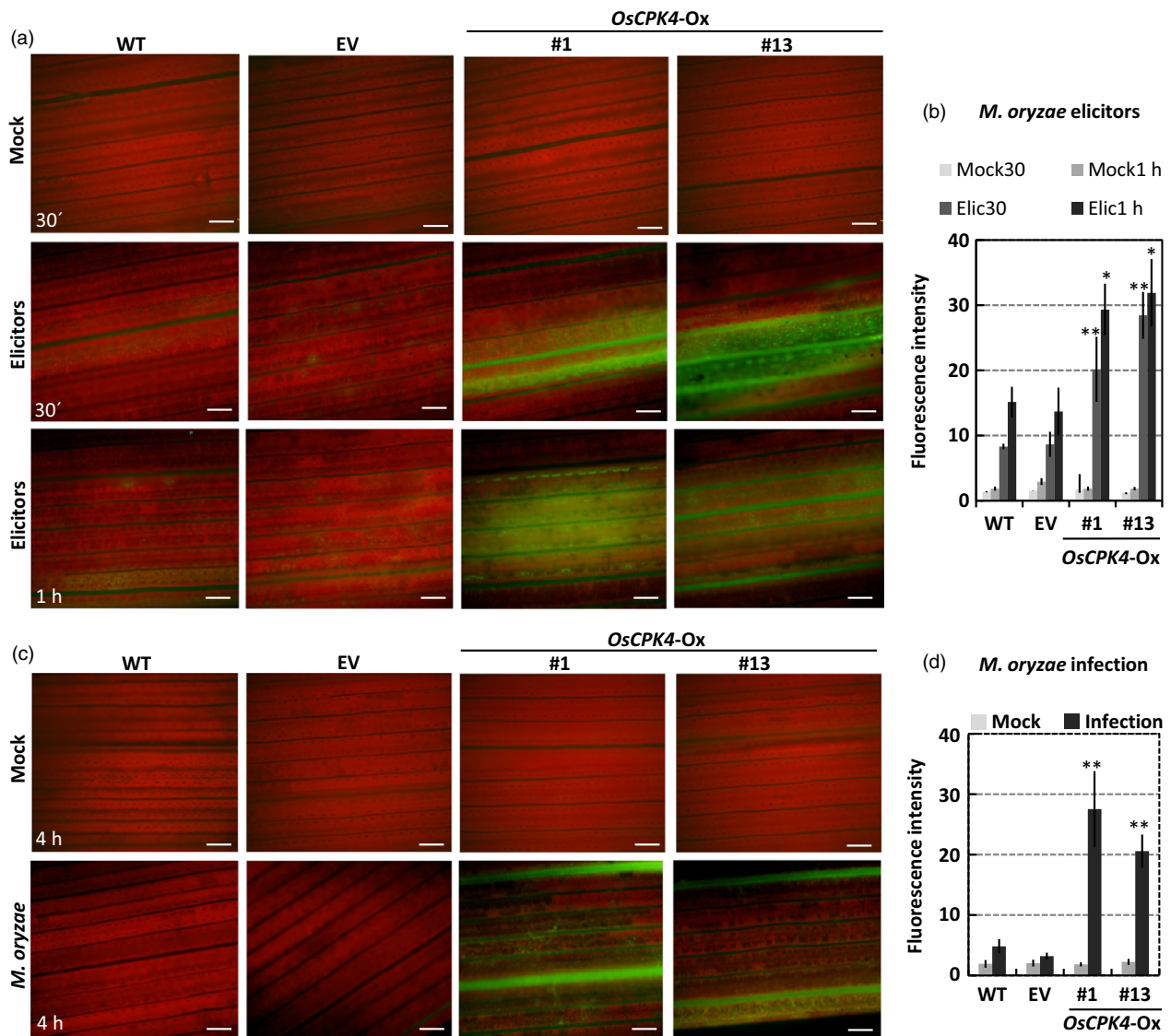


Figure 4 Rapid and strong ROS formation in *OsCPK4*-overexpressing leaves during defence response. Representative epifluorescence microscopy images of wild-type (WT), control empty vector (EV) and *OsCPK4* overexpressor (*OsCPK4-Ox*, lines 1 and 13) leaves after 1-h vacuum infiltration with a 10 μ M CM- H_2 DCFDA solution and treated with (a) *M. oryzae* elicitors (1%) or mock solution; and (c) spore suspension (10^5 spores/mL) or mock solution for the indicated period of time. (b, d) Quantitative comparison of fluorescence intensities in elicitor-treated leaves (b) and fungal-inoculated leaves (d). Values represent the average intensities, and error bars the SD of three independent leaves. Asterisks denote significant differences (one-way ANOVA and Tukey's test, $*P \leq 0.05$, $**P \leq 0.001$). Results are representative of two independent experiments. Scale bar = 200 μ m.

light in the epidermal cell walls of *OsCPK4-Ox* leaves (Figure 5). Quantification of fluorescent leaf area indicated that callose was more abundantly accumulated in the cell walls of *OsCPK4-Ox* leaves inoculated with *M. oryzae* spores (24 hpi) than in noninoculated leaves (Figure 5b). Under the same experimental conditions, callose fluorescence was not detected in control plant leaves. These observations indicate that *OsCPK4* overexpression mediates the constitutive accumulation of callose, and its stronger deposition in response to pathogen infection in rice leaves.

Defence gene expression is potentiated in *OsCPK4* overexpressor rice plants

To further investigate the mechanism underlying *OsCPK4*-mediated disease resistance, the expression profile of rice defence

genes was analysed in the transgenic plants in response to *M. oryzae* infection. First, the expression of the widely used defence marker *OsPBZ1* and *OsPR5* genes was monitored. These genes encode two SA-regulated pathogenesis-related proteins from the PR10 and PR5 families (Datta *et al.*, 1999; Jwa *et al.*, 2006; Midoh and Iwata, 1996; Rakwal *et al.*, 2001). Stronger induction of these two defence genes was observed in *OsCPK4-Ox* plants when compared to wild-type or empty vector control plants upon pathogen challenge (Figure 6a–b). These observations suggest that the *OsCPK4-Ox* plants developed a potentiated defence compared to control plants.

Similarly, the analysis of defence signalling components *OsNPR1/OsNH1* and *OsWRKY45* genes showed a stronger induction in the *OsCPK4-Ox* plants than in the control plants (Figure 6c–d). The two genes encode a transcriptional cofactor

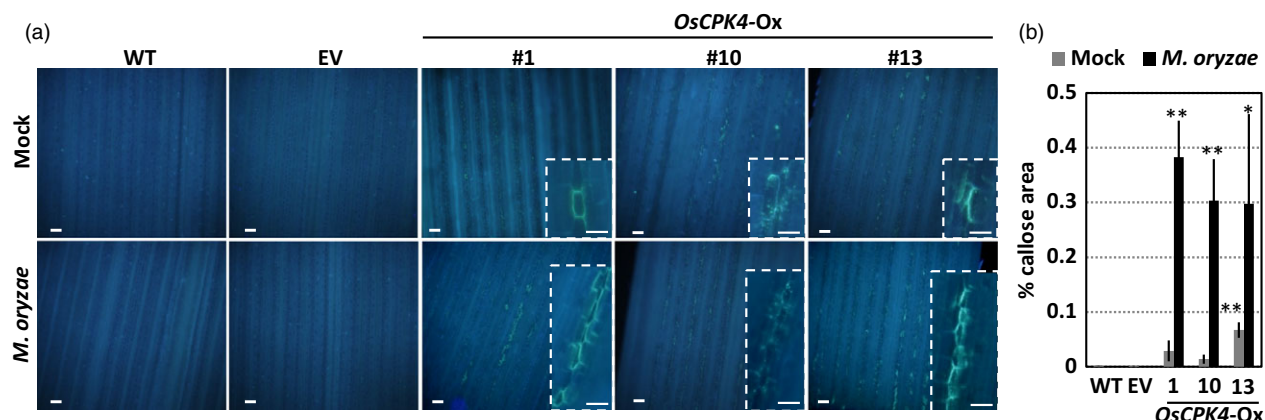


Figure 5 Callose deposition in *OsCPK4*-overexpressing rice leaves. (a) Images of wild-type (WT), empty vector (EV) or *OsCPK4*-overexpressing (*OsCPK4*-Ox) leaves (lines #1, #10 and #13) from 3-week-old plants locally inoculated with *Magnaporthe oryzae* spore suspensions (10^5 spores/mL) or mock solution. Leaves were stained with aniline blue and visualized under UV epifluorescence microscopy at 24 hpi. Magnifications are shown in inset boxes. Bars correspond to 100 μ m, and 50 μ m in inset boxes. (b) Mean values of the percentage of fluorescent area per leaf of three independent replicates per line in three independent assays (a total of 9 leaves per line). Asterisks denote significant differences (one-way ANOVA and Tukey's test, * $P \leq 0.05$, ** $P \leq 0.001$).

and transcriptional factor of the SA-mediated defence pathway (Chern *et al.*, 2001; Shimono *et al.*, 2012). Additionally, upstream components, such as the *OsEDS1* gene encoding an activator of SA signalling (Wiermer *et al.*, 2005), or the *OsSID2* gene encoding the isochlorismate synthase enzyme responsible for part of SA synthesis in plants (Wildermuth *et al.*, 2001), also showed stronger activation in *OsCPK4*-Ox plants (Figure 6e–f). These results show stronger activation of the SA signalling defence pathway in *OsCPK4*-Ox plants that might mediate its enhanced resistance to *M. oryzae*.

Overexpression of *OsCPK4* leads to an increased SA content without compromising rice productivity

The observed strong induction of *OsSID2* gene expression, as well as of other genes related to SA defence signalling, prompted us to quantify the SA content in the *OsCPK4*-Ox lines. We determined the levels of free SA and its glucose conjugate (SAG) under control conditions. No significant differences in free SA levels were detected, but *OsCPK4*-Ox leaves accumulated up to twice as much SAG as compared to the control empty vector or wild-type leaves (Figure 7). Our results indicate that the overexpression of *OsCPK4* leads to the accumulation of SAG in rice leaves under control conditions, which in turn results in the strong activation of downstream SA-mediated defence upon pathogen infection, as revealed by our gene expression studies.

The constitutive accumulation of SA is often associated with disease resistance but is also accompanied by fitness costs, that is a penalty in plant growth and productivity (Takatsui, 2014). To determine the effects of detected high SAG levels in *OsCPK4* rice plants, several fitness parameters of plant growth under controlled conditions were analysed. *OsCPK4*-Ox plants showed a similar appearance than control wild-type and empty vector plants (Figure S3a). They reached the same height at heading time (Figure S3b), flowered at the same period of time after sowing (Figure S3c) and, more importantly, produced a similar grain yield in two different experiments in which plants were grown under random distribution (Figure S3d). Hence, despite the *OsCPK4*-mediated SAG accumulation, our observations indicate that *OsCPK4* overexpression does not have a negative impact in the growth and productivity of rice plants.

Discussion

The present study reveals that the isoform *OsCPK4* from the multigenic family of rice CDPKs has a function in the innate immunity of rice plants. Given that *OsCPK4* was also known to participate in the salt and drought stress responses (Campo *et al.*, 2014), our results demonstrate that *OsCPK4* is a signalling component that positively modulates both abiotic and biotic stress responses in rice plants. This work shows that the expression of the *OsCPK4* gene was rapidly induced in rice leaves when challenged with the *M. oryzae* pathogen and that *OsCPK4* overexpression conferred enhanced resistance to rice blast disease, together supporting that *OsCPK4* mediates the immune response to blast fungus in rice plants. *OsCPK4* accumulation is induced at early stages of the infection process, coinciding with pathogen penetration and suggesting that this protein acts at the earliest signalling events initiated upon pathogen recognition. Among the earliest immune reactions, calcium influxes are included (Blume *et al.*, 2000; Jeworutski *et al.*, 2010; Ranf *et al.*, 2011), which occur through plasma membrane calcium channels activated by the recognition via pathogen recognition receptors (PRRs) of pathogen-associated molecular patterns (PAMPs) (Kurusu *et al.*, 2005). Because *OsCPK4* is localized at the plant plasma membrane (Campo *et al.*, 2014), our hypothesized mechanistic model is that *OsCPK4* acts as calcium sensor of changes stimulated by pathogen perception that triggers the downstream defence signalling events mediated by phosphorylation cascades (Figure 8). In agreement with the proposed mechanism of action, *OsCPK4*-Ox plants that accumulate constitutively increased levels of the protein exhibited a rapid and potentiated defence response upon pathogen infection. These plants accumulate the full *OsCPK4* protein, including the calcium-binding regulatory domain, ready to be stimulated by calcium upon pathogen sensing. Thus, *OsCPK4*-Ox plants showed fast and enhanced ROS production, increased callose deposition and strong defence gene expression when challenged with the *M. oryzae* fungal pathogen. As a result, these plants showed an enhanced disease resistance phenotype against *M. oryzae* as determined by visual inspection, fungal growth quantification and disease lesion measurement. Blast disease resistance was shown

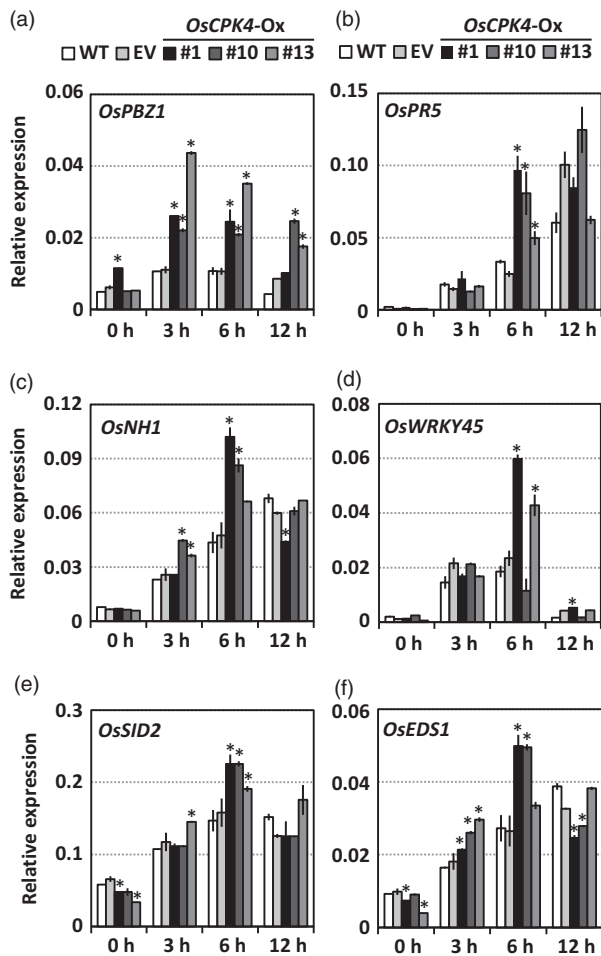


Figure 6 Defence gene expression in *OsCPK4* overexpressor plants in response to *Magnaporthe oryzae* infection. Leaves of wild-type (WT), empty vector (EV) and *OsCPK4*-Ox (lines 1, 10, 13) plants were locally inoculated with a *M. oryzae* spore suspension (10^5 spores/mL) and collected in a pool of 4 leaves at the indicated period of time. Expression levels of indicated defence-related genes were determined by qRT-PCR and normalized to *OsUbi1*. Asterisks denote significant differences (one-way ANOVA and Tukey's test, $P \leq 0.01$). Results are representative of two independent experiments.

not only in detached leaf assays but also in whole-plant infection assays. These results support that *OsCPK4* participates in the signal transmission initiated by pathogen perception, and the constitutive increased accumulation of *OsCPK4* leads to an accelerated and amplified defence signal.

Our results showed that ROS production was stronger and faster in *OsCPK4*-Ox plants upon elicitor or pathogen perception. ROS levels might reach toxic thresholds for *M. oryzae*, leading to fungal penetration blockage as observed under confocal microscopy. However, the importance of ROS in defence reactions is not only due to their toxicity to pathogens, but also to their role as signalling molecules for local and systemic responses (Mittler *et al.*, 2011). ROS mediate the defensive response through oxidative waves that activate signal transduction through phosphorylation cascades, accompanied by hormonal signalling and the expression of defence-related genes (Baxter *et al.*, 2013; Shetty *et al.*, 2008). Therefore, the increased ROS production might contribute to the enhanced defence responsiveness

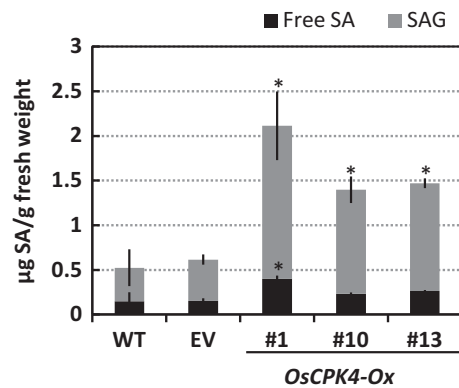


Figure 7 Increased content of total SA, free SA and glucoside conjugate (SAG) in *OsCPK4* overexpressor plants. Data are mean values of two independent quantification in a pool of 3 leaves from 3-week-old wild-type (WT), empty vector (EV) or *OsCPK4* overexpressor (*OsCPK4*-Ox) plants. Asterisk denotes significant differences (one-way ANOVA, $P < 0.05$).

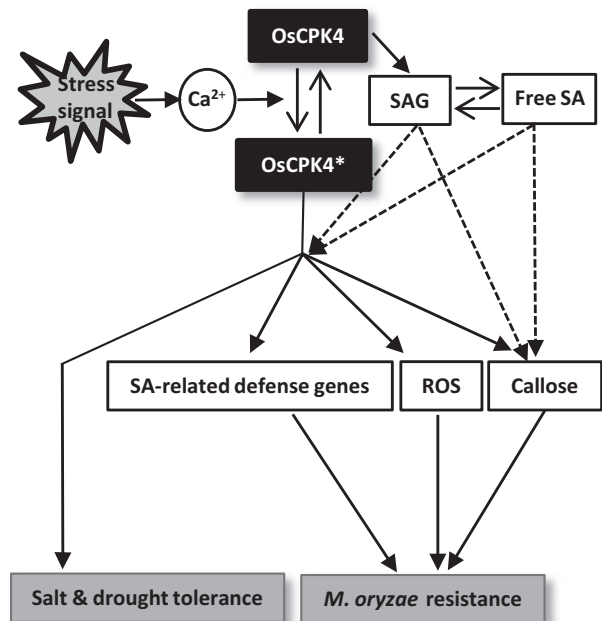


Figure 8 Model for *OsCPK4*-mediated defence responses. Stress induces Ca^{2+} increase that activates *OsCPK4* leading to ROS production, callose deposition and SA-regulated defence gene expression resulting in resistance to *Magnaporthe oryzae* infection. *OsCPK4* also mediated the accumulation of SAG.

observed in *OsCPK4*-Ox plants. Be as toxic compound or as signalling molecules, ROS production seems to contribute to the enhanced resistance of *OsCPK4*-Ox plants, and to be activated by *OsCPK4* in response to PAMP stimulation. Connections between ROS production and CPKs have been already described in the literature; these studies report that ectopic expression of constitutively active CPK variants resulted in an increased production of ROS (Dubiella *et al.*, 2013; Kobayashi *et al.*, 2007; Romeis *et al.*, 2001; Xing *et al.*, 2001). Moreover, NADPH oxidases playing a central role in the oxidative burst during immune responses have been reported as CPK targets in potato and Arabidopsis (Dubiella

et al., 2013; Kobayashi *et al.*, 2007). Similarly for rice, the plasma membrane NADPH oxidases might be potential targets of the plasma membrane-associated OsCPK4 protein, triggering a fast and strong oxidative burst upon pathogen attack in the plants that constitutively accumulated increased levels of OsCPK4 protein. Other sources for ROS production also exist in plant cells, such as the peroxidases identified in Arabidopsis as major contributors to ROS production during responses to fungal elicitors (Daudi *et al.*, 2012), and they might be also potential OsCPK4 targets. Future studies will address OsCPK4 target identification.

OsCPK4 overexpressor plants accumulate increased SAG levels, the glycosylated form of SA. SAG is considered a likely storage form of physiologically active free SA, which is accumulated in the vacuole to serve as a source of free SA when required in dicotyledonous plants (Dean *et al.*, 2005; Seo *et al.*, 1995). In rice plants, SAG has been proposed to have *per se* a role in activating defences for induced resistance (Umemura *et al.*, 2009). This increased accumulation of SAG prepared OsCPK4-overexpressing rice plants for a strong activation of SA-mediated defence signalling upon *M. oryzae* infection. As a result, intense activation of components of the SA pathway was detected, including the biosynthetic gene *OsSID2*, the *OsNH1* and *OsWRKY45* transcriptional activator genes and the end products *OsPBZ1* and *OsPR5*. Another immune response associated with SA is the callose deposition, being promoted by SA (Yi *et al.*, 2014). In agreement with the high SAG content, callose was also accumulated in the OsCPK4-Ox. Callose might represent a physical barrier that prevents fungal penetration leading to the observed resistant phenotype of OsCPK4-Ox plants. Our results reveal that OsCPK4 contributes to the accumulation of SAG and callose in rice plants under noninductive conditions.

Our data suggest that the rice plants overexpressing OsCPK4 are sensitized or preconditioned for a robust and fast immune response by accumulating a signalling component that can be immediately activated upon exposure to stress. Defence responses usually have fitness costs associated with resource allocation for defensive compounds or the toxicity of the defensive products (van Hulten *et al.*, 2006), and the strategies to improve disease resistance in plants based on the constitutive activation of defences are accompanied by negative effects on plant growth and yield (Gust *et al.*, 2010; Takatsuji, 2014). In this sense, we have shown that the overexpression of the OsCPK4 gene in rice plants does not have a negative impact on plant performance, at least under containment conditions. The growth, flowering time and yield fitness parameters of these plants are not significantly different than those of the wild-type plants. This is in agreement with the observation that OsCPK4-overexpressing rice plants did not show the constitutive expression of defence-related genes or ROS accumulation under noninductive conditions, although they do accumulate SAG and callose. This is consistent with the already reported global transcriptomic analyses showing that overexpression of OsCPK4 in rice plants has a low impact in the rice transcriptome (Campo *et al.*, 2014). Altogether, our results support that OsCPK4 might be a good target for blast protection while maintaining rice yield.

OsCPK4-Ox rice plants are also more tolerant to salt and drought stress (Campo *et al.*, 2014). SA, in addition to modulate the immune response in plants, is also known to improve the tolerance to salt and drought stress by preventing membrane damage among other mechanisms (Farooq *et al.*, 2009; Jayakannan *et al.*, 2013). Moreover, SA inhibits lipid peroxidation, thus protecting cell membranes (Dinis *et al.*, 1994; Lapenna

et al., 2009). Therefore, the improved tolerance to drought and salinity of OsCPK4-Ox rice plants associated with a reduction in lipid peroxidation could be mediated by the increased content of SAG. This is an interesting result because trade-offs between defence and abiotic stress tolerance have been frequently reported (Sharma *et al.*, 2013). For instance, OsCPK12 oppositely modulates salt stress tolerance and blast disease resistance (Asano *et al.*, 2012). However, crosstalk between biotic and abiotic signalling pathways can result not only in negative but also in positive functional outcomes (Sharma *et al.*, 2013). Our studies demonstrate that OsCPK4 acts as a convergence component that positively modulates both biotic and abiotic signalling pathways, presumably modulating SA levels and suggesting that it is a good molecular target to improve tolerance to different stresses in rice plants.

Experimental procedures

Plant and fungal growth conditions

OsCPK4 overexpressor rice plants were previously generated and described (Campo *et al.*, 2014). They were grown at 28 °C with a 14-h/10-h light/dark photoperiod. Fungal strains of *M. oryzae* FR13 isolate (provided by D. Tharreau, CIRAD Montpellier, France) and Guy11-GFP (provided by A. Sesma, CBGP Madrid, Spain) were grown in oatmeal agar (72.5 g/L, 30 mg/L chloramphenicol) for 2 weeks at 28 °C using a 16-h/8-h light/dark photoperiod. Their spores were collected in sterile water, filtrated with Miracloth (Calbiochem) and adjusted to the appropriate concentration using a Bürker counting chamber. *M. oryzae* elicitors were obtained as previously described (Casacuberta *et al.*, 1992). *F. verticillioides* and *D. dadantii* strains were grown as previously described (Gómez-Ariza *et al.*, 2007).

RNA isolation and RT-qPCR

Gene expression levels were determined from a pool of four leaves at the same developmental stage of 3-week-old soil-grown plants. Total RNA was extracted using TRIzol reagent (Invitrogen, Basel, Switzerland). DNase-treated RNA (1 µg) was retrotranscribed using the transcriptor first cDNA synthesis kit (Roche, Mannheim, Germany). qRT-PCR analyses were carried out in 96-well optical plates in a LightCycler® 480 System (Roche) according to the following programme: 10 min at 95 °C, 45 cycles of 95 °C for 10 s and 60 °C for 30 s and an additional cycle of dissociation curves to ensure a unique amplification. The reaction mixture contained 5 µL of SYBR Green Master mix reagent (Roche), 2 µL of 1:4 diluted cDNA sample and 300 nM of each gene-specific primer (Table S1) in a final volume of 10 µL. The results for the gene expression were normalized to *OsUbi1* (LOC_Os06g46770) and *OsUbi5* (LOC_Os01g22490) genes as indicated. Three technical replicates were performed for each sample.

Protein extracts, CPK activity and immunoblot analysis

Protein extracts were obtained from membrane-enriched fractions prepared from leaves in a pool of at least four plants. Samples were ground in liquid nitrogen, thawed in two volumes of extraction buffer (10% sucrose, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 1 mM PMSF) and centrifuged at 15 000 g for 20 min at 4 °C. The pellet was resuspended in 2 volumes of elution buffer (1% Triton X-100, 25 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1 mM PMSF) using a cooled sonication bath. Protein extracts were recovered from the supernatant after centrifugation as before, quantified, separated

in SDS-PAGE and transferred to nitrocellulose membranes. Western blot analyses were performed using anti-OsCPK4 antibodies as described (Campo *et al.*, 2014). Two independent experiments with 3 biological repeats on a pool of 3 independent plants for each time point were analysed. Antibodies were raised against the N-terminal variable domain of OsCPK4 (Met1 to Arg58) to specifically recognize this isoform of the conserved OsCPK family protein.

The calcium-dependent kinase activity was analysed as described with minor modifications (Boudsocq *et al.*, 2012). These include that total protein was extracted from rice leaves and immunoprecipitated for 2 h with specific anti-OsCPK4 antibodies bound to Dynabeads® with the antibody coupling kit (Life Technologies, Carlsbad, CA, USA), that the phosphorylation substrates were β -casein peptide (Sigma St. Louis, MO, USA) and myelin basic protein (Invitrogen) and that the unincorporated radioactive nucleotides were discarded using MicroSpin G-25 columns (GE Healthcare Little Chalfont, UK). The concentration of free calcium in each buffer was calculated using MaxChelator (<http://maxchelator.stanford.edu/>).

Disease resistance assays with rice pathogens

M. oryzae infections were performed using a detached leaf infection assay as described (Coca *et al.*, 2004), or a whole-plant infection assay by spraying the fungal spores with an aerograph at 2 atmospheres of pressure. Infection assays were carried out with 3-week-old plants grown in soil, using three pots with 10 plants each per line and 2 mL of spore suspension (10^5 spores/mL) per pot. The plants were maintained for 16 h in a closed plastic bag for high humidity conditions after inoculation. Lesion areas were measured by image analysis software Assess v.2.0 at 7 days postinoculation (dpi). Fungal biomass in rice infected leaves was determined at 7 dpi by qPCR using specific primers for the 26S ribosomal RNA gene of *M. oryzae* and normalized to *OsUbi1* gene as described (Qi and Yang, 2002). DNA (15 ng per qPCR) was obtained from the rice infected leaves as described (Murray and Thompson 1980), but using MATAB as extraction buffer (0.1 M Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% MATAB, 1% PEG 6000 and 0.5% sodium sulphite). Disease symptoms on whole-plant infection assays were scored at 7 dpi following the Standard Evaluation System for blast rice disease (IRRI, 2002). Three biological replicates were performed for each line and three technical replicates per sample.

Infection assays with *F. verticillioides* were performed as previously described with minor modifications (Bundó *et al.*, 2014), including a seed germination period of 8 h previous inoculation with 10^3 spores/mL suspensions.

Assays with *D. dadantii* were carried out as described with minor modifications (Gómez-Ariza *et al.*, 2007), reducing the seed germination period to 8 h and increasing the inoculation doses to 10^7 CFU.

Fluorescence microscopy

Confocal laser scanning microscopy was performed using an Olympus FV1000 microscope (Tokyo, Japan). GFP was excited with an argon ion laser emitting at 488 nm and fluorescence detected at 500–550 nm. Chlorophyll autofluorescence was visualized at 600–700 nm. Lesions were also observed under a Zoom Stereo Microscope Olympus SZX16 fitted with an Olympus DP72 digital camera.

For ROS detection, leaf segments from at least three different plants were infiltrated with a 10 μ M solution of the fluorescent probe CM-H₂DCFDA (Molecular Probes) in 100 mM phosphate buffer pH 7.2 for 2 h. The leaves were then treated with a 1% *M. oryzae* elicitor solution in sterile water or inoculated with a 10^5 spores/mL suspension. ROS was monitored over the time using an Axiophot Zeiss epifluorescent microscope, and fluorescent signals were quantified by image analysis using the ImageJ software.

Callose accumulation was visualized by fluorescence under epifluorescence microscopy after aniline blue staining of leaf segments from at least three different plants as previously described (Luna *et al.*, 2010). The fluorescent area per leaf segment was quantified also using the ImageJ software.

Salicylic acid quantification

Free SA and SA β -glucoside (SAG) content in rice leaves was determined as previously described with some minor modifications (Coca and San Segundo, 2010). Total SA was obtained from 1 g of fresh grinded leaves by two consecutive methanol and ethanol extractions (3 mL each). After alcohol evaporation, the extracts were resuspended in water and separated into two parts, one to determine free SA and the other for SAG. SAG samples were digested with 10 U/mL of β -glucosidase from almonds (Sigma) at 37 °C during 16 h. After digestion, the samples were filled up to 1 mL with milli-Q water, and HCl 37% (50 μ L) was added. They were subjected to two consecutive extractions with ethyl acetate/cyclopentane/isopropanol (2 mL, 50:50:1). Organic phases were evaporated and resuspended in methanol (25 μ L) for the HPLC analysis using a Zorbax Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA, USA). Two biological replicates were performed for each independent line.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1 Primers used for gene expression analysis.

Figure S1 Expression of *OsCPK4* gene, protein accumulation and CPK activity in the transgenic rice leaves.

Figure S2 Susceptibility of *OsCPK4*-overexpressing seedlings to *Fusarium verticillioides* and *Dickeya dadantii* rice pathogens.

Figure S3 Plant performance of *OsCPK4*-overexpressing rice plants